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ORIGINAL PAPER

Identification of heavy metal-induced genes encoding glutathione S-transferases in the arbuscular mycorrhizal fungus *Glomus intraradices*

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Abstract Arbuscular mycorrhizal fungi are able to alleviate the stress for plants caused by heavy metal contamination of soil. To analyze the molecular response of arbuscular mycorrhizal fungi to these pollutants, a subtractive cDNA library was constructed using RNA from *Glomus intraradices* extraradical hyphae of a root organ culture treated with a mixture of Cd, Zn, and Cu. Screening by reverse Northern blot analysis indicated that, among 308 clones, 17% correspond to genes up-regulated by heavy metals. Sequence analysis of part of the clones resulted, amongst others, in the identification of six genes putatively coding for glutathione S-transferases belonging to two different classes of these enzymes. Expression analyses indicated that the genes are differentially expressed during fungal development and that their RNA accumulation dramatically increases in extraradical hyphae grown in a heavy metal-containing solution.

Keywords Extraradical hyphae · *Glomus intraradices* · Heavy metal · Gene expression · Root organ cultures

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Introduction

Besides having a beneficial effect in other adverse soil conditions (Smith and Read 1997; Jeffries et al. 2003), numerous studies have revealed that arbuscular mycorrhizal (AM) fungi confer plants with tolerance against heavy metal (HM) stress (e.g., El-Kherbawy et al. 1989; Heggo and Angle 1990; Davies et al. 2001). Different mechanisms are proposed to be involved in this activity (Meharg 2003). Binding of cations to the extraradical hyphae seems to play a role (Galli et al. 1994; Joner et al. 2000) and analyses of development of AM fungi under HM stress suggest such an avoidance strategy (Pawlowska and Charvat 2004). Other experiments show that HMs can be transported by AM fungi into the roots, where they seemed to be retained in fungal structures and not transferred to the plant cells (Joner and Leyval 1997; Kaldorf et al. 1999; Tonin et al. 2001). Changes in the community of HM oxidizing and reducing bacteria from the rhizosphere to the mycorrhizosphere have also been observed (Nogueira et al. 2004). To understand the mechanisms of HM tolerance on the plant side, the influence of species or cultivars with different root densities has been analyzed (Joner and Leyval 2001; Rivera-Becerril et al. 2002) and changes in plant protein or gene expression patterns monitored (Repetto et al. 2003; Rivera-Becerril et al. 2005). Concerning the fungal side, strains were isolated from many different contaminated soils (Sambandan et al. 1992; Turnau et al. 2001), and further experiments indicate that such isolates are better adapted to HM stress than those from noncontaminated soils (Weissenhorn et al. 1994; Diaz et al. 1996; Del Val et al. 1999; Hildebrandt et al. 1999; Malcova et al. 2003a). To produce inocula, which could be used for revegetation, AM fungal isolates were cultivated under different conditions. These experiments showed that the ability to confer HM tolerance decreases after a few

generations if inoculum production is carried out on HM-free substrates (Rydlova and Vosatka 2003; Malcova et al. 2003b). On the other hand, AM fungi treated with cadmium for only two generation cycles showed an improved prevention of Cd movement in the plant compared to the corresponding controls (Tullio et al. 2003).

Differences between AM fungal isolates in conferring HM tolerance, as well as loss or gain of this capacity, are probably based on genome variation and flux, and/or on differential gene expression. Understanding the molecular basis of these phenomena requires isolation and analysis of genes whose products are involved in the responsible mechanisms. Metallothioneins are such gene products and the corresponding genes have been detected in cDNA libraries of the AM fungi *Gigaspora rosea* and *Gigaspora margarita* (Stommel et al. 2001; Lanfranco et al. 2002). More detailed experiments showed that the *G. margarita* gene confers HM tolerance, if it is expressed in yeast (Lanfranco et al. 2002). To obtain a broader view of the mechanisms of AM fungi in responding to HMs, we decided to identify genes induced by these soil contaminants. We chose *Glomus intraradices* for our experiments because it has been foreseen as an ideal model system for AM fungal research due to its small genome size (Hijri and Sanders 2004). A subtractive cDNA library was established from RNA of extraradical hyphae which were treated or not with an HM mixture. This library was analyzed and a gene family encoding glutathione S-transferases (GSTs) was studied for its RNA accumulation pattern.

Materials and methods

AM fungus culturing conditions

The AM fungus *G. intraradices* (Schenck and Smith) DAOM 181602 was grown in sterile culture with Ri-transformed roots of two different host plants, *Daucus carota* L. and *Medicago truncatula* Gaertn. A17. A split Petri dish system was used for the analysis of extraradical hyphae (St-Arnaud et al. 1996). After 6 weeks of growth of the carrot cultures at 25°C in solid M-medium, the hyphal compartment of the Petri dish system was depleted and liquid M-medium without sucrose was supplied. In two plates, the M-medium was enriched with 4.5 μM $CdCl_2$, 50 μM $ZnCl_2$, and 75 μM $CuCl_2$ (+HM treatment). Two other plates remained untreated as control plates. The newly grown hyphae were harvested after 2 weeks with a scalpel and fine forceps and immediately used for RNA extraction. The *M. truncatula* root organ cultures were harvested after 2 months for obtaining extraradical hyphae, mycorrhiza, and spores (Doner and Bécard 1991). The latter were germinated in sterile water before RNA extraction (Tamasloukht et al. 2003).

RNA extractions and cDNA subtractive hybridization

The RNA was extracted using the RNeasy Plant Mini Kit including the on-column-DNase treatment (Qiagen, Hilden, Germany) following the protocol of Requena et al. (1999). The quality and quantity of the RNA was measured in the Bioanalyzer 2100 with the corresponding RNA Lab Chip Kit (Agilent Technologies, Boeblingen, Germany). The cDNA was synthesized from 250 ng RNA per sample using the Super-SMART cDNA synthesis kit (BD Bioscience Clontech, Heidelberg, Germany). A PCR with 18 cycles was determined as the optimal condition for the second strand synthesis. The establishment of the cDNA population enriched for HM-induced genes was carried out by suppressive subtractive hybridization (SSH, Diatchenko et al. 1996) according to the instructions of the Clontech PCR-Select cDNA Subtraction Kit (BD Bioscience Clontech). The SSH products were amplified, cloned in the pGEM Teasy vector (Promega, Mannheim, Germany) and transformed in *Escherichia coli* DH 5 alpha cells (Invitrogen, Karlsruhe, Germany).

Expression pattern analysis of HM-induced genes

To analyze RNA accumulation in HM-treated and control hyphae, reverse Northern blots were carried out in two parallel experiments as described by Tamasloukht et al. (2003). Briefly, inserts of the SSH library were amplified, separated in parallel on two agarose gels, and transferred onto nylon membranes. Each membrane carried in addition a fragment of the *GintTef*1 gene (AJ831587) for calibration of the hybridization signals. The membranes were subsequently hybridized to double-stranded cDNA probes synthesized with the Super-SMART kit, incorporating digoxigenin-labeled deoxyribonucleotide triphosphates (Roche, Penzberg, Germany) during second strand synthesis. Double-stranded SMART cDNAs were also used as templates for quantitative real-time PCR experiments applying an annealing temperature of 52°C for all *Gst* primer pairs and 58°C for the *Tef*1 primer pair (Table 2). The experiments were carried out in 25-μl volume using the QuantiTect™ SYBR® Green PCR Kit (Qiagen) with 2 μl SMART cDNA as template and 0.15-μM prime pairs. A dilution series of 10^8 to 10 molecules of the fragments was used. Detection of the amplified fragments and recording of the melting curve was done on an iCycler (Bio-Rad, Muenchen, Germany) with 15 min at 95°C and 45 cycles (30 s at 95°C, 30 s at the annealing temperature, 30 s at 72°C) followed by heating from 55 to 95°C with a rate of 0.5°C per 10 s. The copy number was calculated with the computer program iCycler iQ-System version 2.1.A (Bio-Rad). All reactions were performed with three replicates. The results were statistically analyzed with the Mann–Whitney U-test.

Sequence analyses and comparisons

After sequence analysis of the clones from the SSH library (MWG-Biotech, Ebersberg, Germany), similarity searches of the inserts were carried out with BlastX (Gish and States 1993) and BlastN (Altschul et al. 1990) on March 29, 2006. Nucleotide and deduced amino acid sequence alignments were carried out with ClustalW (Thompson et al. 1994). The phylogeny of the amino acid sequences was reconstructed by quartet puzzling using the program PUZZLE, carrying out 1,000 replicates (version 4.0.2, Strimmer and von Haeseler 1996), and the results were displayed as dendrogram with the program TREEVIEW (version 1.5.3, Page 1996).

Results

HM-induced genes from *G. intraradices*

To identify genes induced by Cd, Zn, and Cu, an SSH library was constructed using RNA from 2-week-old HM-treated and control extraradical hyphae of an AM symbiosis between carrot and *G. intraradices* established in root organ cultures. Three hundred eight clones were collected in microtiter plates and expression of the corresponding genes was monitored by reverse Northern blot analysis (example in Fig. 1). This suggested that 52 of the inserts belonged to HM-induced genes in the extraradical mycelium of *G. intraradices*. Sequencing and annotation resulted in 31 different cDNA sequences. Fourteen fragments revealed significant similarities to proteins with known function and one showed similarity to a predicted protein (Table 1). Twelve fragments overlapped with expressed sequence tags (ESTs) from other cDNA libraries of *G. intraradices* and therefore probably belong to identical genes (Table 1). Twenty-one ESTs of HM-induced genes showed no similarities to entries in the databases (AJ574648, AJ574649, AJ574652, AJ574654, AJ574657, AJ574658, AJ574674, AJ574677, AJ574678, AJ574679, AJ574686, AJ574698, AJ574713, AJ574718, AJ574727, AM168261, AM168262, AM168265, AM168266, AM168268, and AM168269).

GST gene family of *G. intraradices*

Among the cDNA fragments belonging to HM-induced genes, six different genes putatively encoded the carboxyl terminus of GSTs (*GintGst*1–6 in Table 1). *GintGst*1 showed an overlap of nearly 100% identity with an EST from *G. intraradices* extraradical hyphae (AUO82837, Sawaki and Saito 2001) and *GintGst*3, *GintGst*4, and *GintGst*6 with cDNA sequences from germinated spores

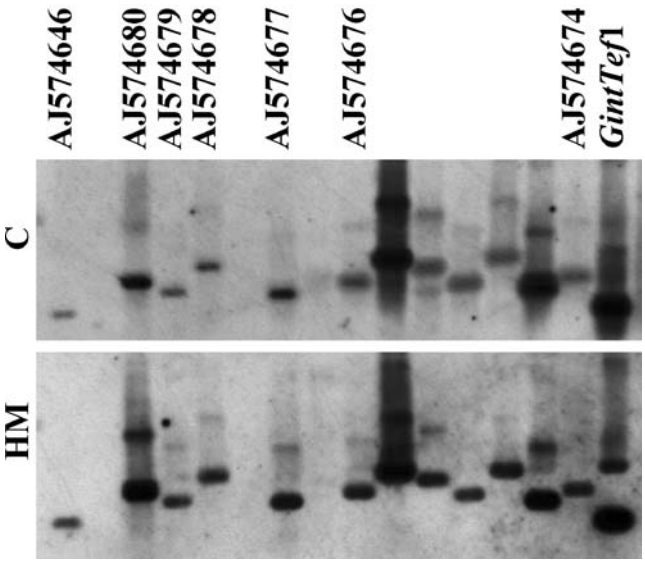


Fig. 1 Reverse Northern blot analysis. cDNA fragments from the suppressive subtractive library of *G. intraradices* extraradical hyphae were PCR amplified, separated into two parallel experiments by gel electrophoresis, transferred onto nylon membranes and hybridized to complex cDNA probes derived from RNA of HM-treated extraradical hyphae (*HM*) or control hyphae (*C*). The figure shows an example of 15 analyzed fragments together with a fragment of the constitutively expressed gene *GintTef*1. Accession numbers are indicated for those considered as being regulated and sequenced

(BM439293: Podila et al. unpublished; BM959570: Bago et al. 2002; BM027594: Podila et al. unpublished). Two ESTs (AJ574728 and AM168258) showed similarity to the 5′ end of GST genes and also overlapped with the cDNA sequence BM959570, which suggested they belong to *GintGst*4. This was verified by PCR (data not shown). *GintGst* sequences were assembled from the ESTs of this study and the cDNA sequences deposited in the database. *GintGst*1–5 showed nucleotide similarities of between 78 and 90% in their coding regions. One (*GintGst*6), however, was different with only 45% identical nucleotides. Similarity searches showed that the deduced amino acid sequences GINTGST1–5 are most closely related to different plant GSTs, while the putative GINTGST6 revealed the highest number of identical residues with a protein from the fungus *Botryotinia fuckeliana*. To group these sequences from *G. intraradices* into the different classes of fungal GSTs, they were compared with proteins from a recently published phylogenetic analysis (McGoldrick et al. 2005). This suggested that the putative GST1–5 belong to the phi class and GST6 to the cluster 2 of this protein superfamily (Fig. 2).

RNA accumulation analyses of GST genes

To analyze the RNA accumulation pattern of the six different GST genes, primers were designed based on the

Table 1 ESTs from *G. intraradices* with significant similarities to HM-induced genes. ESTs are shown which gave stronger signals after hybridization to a complex cDNA probe derived from extraradical hyphae treated with a mixture of HMs compared to the corresponding control cDNA probe. The accession number, the length, and the result of a BlastX search with *e* values lower than e^{-5} are indicated for each *G. intraradices* EST. The fifth column shows accession numbers of *G. intraradices* sequences in databases, which show a 100% identity to the ESTs, while the last column indicates the *GintGst* gene number

AC number	Length (nt)	Sequence similarity (BlastX)	Significance/identical aa	Overlapping with	*Gint Gst*
AJ574645	527	GST *Arabidopsis thaliana*	$1e^{-18}$/42%	–	2
AJ574660					
AJ574750					
AJ574646	284	Histone deacetylase *Schizosaccharomyces pombe*	$4e^{-7}$/36%	–	
AJ574647	420	–	–	AU098221[a]	
AJ574650	292	Predicted protein *Neurospora crassa*	$7e^{-12}$/63%	–	
AJ574651	575	GST *Glycine max*	$6e^{-12}$/39%	BM439293[b]	3
AJ574689					
AJ574690					
AM168256					
AM168259					
AM168263					
AJ574653	614	aldo/keto reductase *Deinococcus radiodurans*	$2e^{-57}$/60%	CF200382[b]	
AJ574655	207	DnaJ-like protein *Nicotiana tabacum*	$1e^{-14}$/65%	–	
AJ574656	533	GST *Arabidopsis thaliana*	$6e^{-17}$/40%	AU082837[a]	1
AJ574661	513	GST *Botryotinia fuckeliana*	$5e^{-14}$/44%	BM027594[b]	6
AJ574662	636	–	–	BM959461[c]	
AJ574675	631	Guanosine diphosphatase *Candida albicans*	$4e^{-47}$/46%	–	
AJ574676	425	Phosphoenolpyruvate carboxykinase *Saccharomyces cerevisiae*	$7e^{-66}$/80%	AL388327[d]	
AJ574680	487	GST *Arabidopsis thaliana*	$2e^{-15}$/37%	–	5
AJ574697	305	Thioredoxin *Aspergillus nidulans*	$8e^{-9}$/52%	CF200334[b]	
AJ574700	255	–	–	CF200468[b]	
AM168257					
AJ574728	227	GST *Arabidopsis thaliana*	$7e^{-8}$/50%	BM959570[c]	4
AM168258					
AJ574758	523	GST *Glycine max*	$7e^{-17}$/40%	BM959570[c]	4
AM168260	532	Glyoxal oxidase *Botryotinia fuckeliana*	$3e^{-29}$/39%	–	
AM168264					
AM168267	339	–	–	AC156585[e]	

[a] EST from a cDNA library of extraradical hyphae (Sawaki and Saito 2001)
[b] EST from a cDNA library of germinating spores (Podila et al. unpublished)
[c] EST from a cDNA library of germinating spores (Bago et al. 2002)
[d] EST from a mycorrhiza cDNA library (Journet et al. unpublished)
[e] Genomic clone (Department of Energy Joint Genome Institute unpublished)

known sequences (Table 2). PCR experiments with the different primer pairs using the corresponding clones as templates showed that these primers are specific concerning at least the identified genes (data not shown). Expression at different stages of fungal development was analyzed by real-time PCR using RNA from germinating spores, extraradical hyphae, and mycorrhiza of 2-month-old *M. truncatula* root organ cultures (Fig. 3a). Except for *Gst*5, the genes showed a higher RNA accumulation level in the extraradical hyphae compared with the other developmental stages. In addition *Gst*1, *Gst*4, and *Gst*5 seemed to be more transcribed in germinated spores than in intraradical hyphae, while *Gst*3 showed the opposite expression pattern. The differences were, however, low.

For a second series of real-time PCR experiments, extraradical hyphae were either treated or not with the HM mixture for 2 weeks, as described above (Fig. 3b). RNA was extracted and used in real-time PCR experiments with the six primer pairs. In general, the expression was much lower in these young extradical hyphae compared to those from the 2-month-old root organ cultures (Fig. 3a). All genes were, however, dramatically induced upon treatment with HMs. Only *Gst*2 showed a relatively low induction level of approximately fivefold.

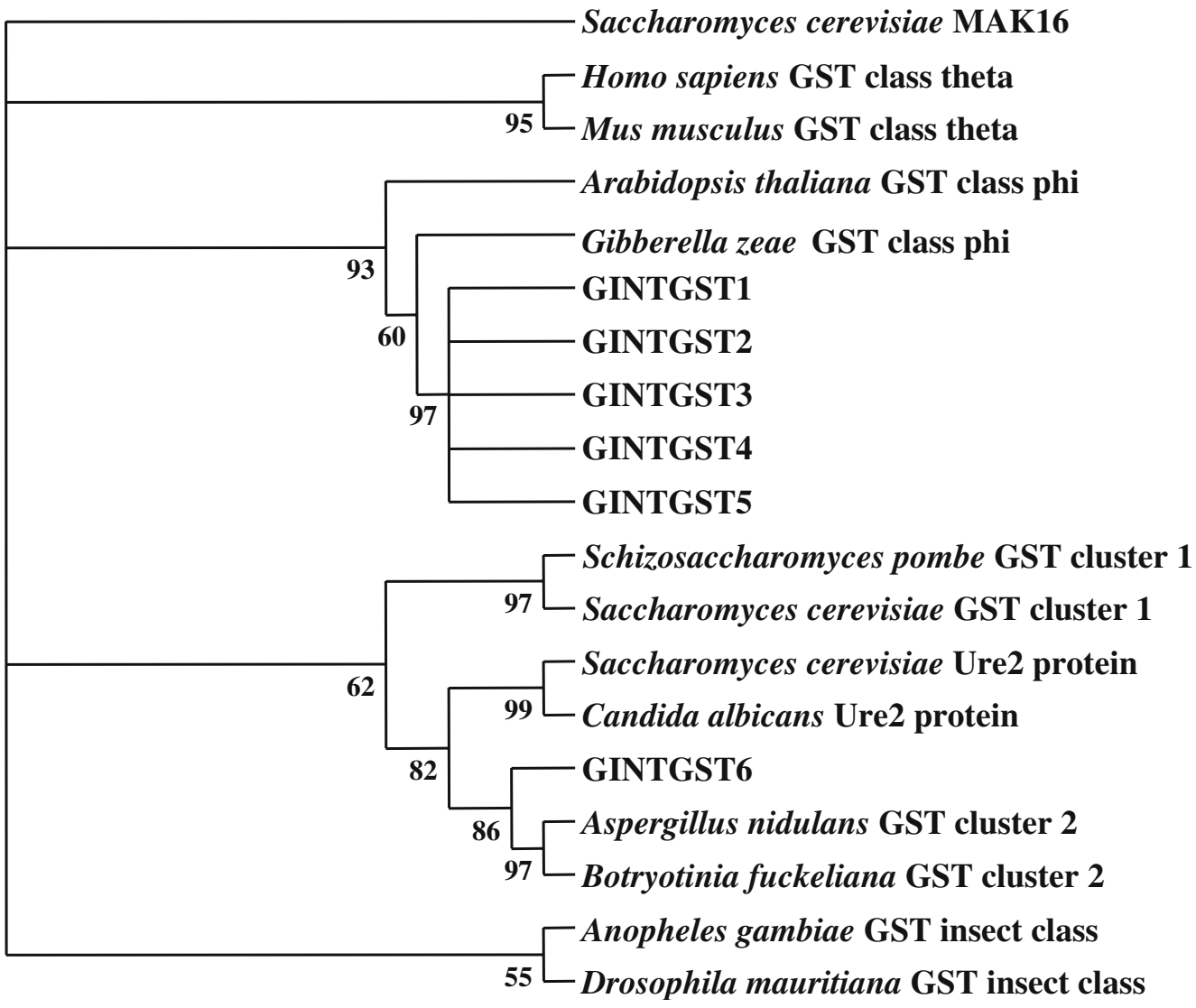


Fig. 2 Phylogenetic analysis of GSTs. Deduced amino acid sequences of GINTGST1 to GINTGST6 from *G. intraradices* (number of residues: 159, 122, 150, 203, 125, and 126) were aligned using ClustalW with members of the GST-like protein superfamily: Ure2 proteins from *Saccharomyces cerevisisae* (354 aa, NP_014170) and *Candida albicans* (355 aa, Q8NJR5), cluster 1 GSTs from *Schizosaccharomyces pombe* (242 aa, Q9P6M1) and *S. cerevisisae* (234 aa, NP_012304), phi class GSTs from *G. zeae* (217 aa, XP_391216) and *A. thaliana* (215 aa, Q96266), theta class GSTs from *Homo sapiens* (242 aa, AAC13317) and *Mus musculus* (244 aa, AAB03534), insect GSTs from *Drosophila mauritiana* (200 aa, P30105) and *Anopheles gambiae* (224 aa, AAL59658), cluster 2 GSTs from *B. fuckeliana* (254 aa, AAG43132) and *A. nidulans* (250 aa, AAM48104), and the MAK16 protein from *S. cerevisisae* as outgroup (306 aa, NP_009377). Based on the alignment, the phylogeny of the putative proteins was reconstructed with the program PUZZLE and the distance tree was displayed by the program TREEVIEW. Quartet puzzling support values of 1,000 replicates are indicated

Discussion

Fragments of protein-encoding AM fungal genes were first detected in cDNA libraries from mycorrhiza or were obtained by targeted PCR cloning using genomic DNA or total RNA from spores (for review, see Franken and Requena 2001). More recently, cDNA libraries have been constructed from pure fungal material and used for establishing collections of expressed sequence tags (for review, see Ferrol et al. 2004). Genes putatively involved in the response to HMs have been identified in these EST libraries and such a role could be verified for two of them encoding a metallothionein and a zinc transporter (Lanfranco et al. 2002; González-Guerrero et al. 2005). To obtain an EST library enriched for ESTs of HM-regulated genes of *G. intraradices*, we used SSH. This technique has been successfully applied to identify genes differentially expressed during AM fungal development

Table 2 Primers used to analyze RNA accumulation of the five GST encoding genes and of the translation elongation factor EF-1alpha encoding gene of *G. intraradices* by reverse transcriptase and real-time PCR

Primer	Sequence
GintGst1.for	CTT CTT ATT ACA CTC AAC ATT TAG
GintGst1.rev	TTA CAG CTT ATA AAA TAG AGT AT
GintGst2.for	TTG GTT GGG CAA TTA ATT TTC GC
GintGst2.rev	AAG CAT ACT ATT TAT CAC AAT G
GintGst3.for	TTC CAC CTC TAT TTA AAT TGC TTA C
GintGst3.rev	TTA CAA AGA ATA AGC TAC TTA TC
GintGst4.for	CTC TTA TTA TAG TCC ACA TTT AAG
GintGst4.rev	GCA ATT GCT TAC ACA GTA TAA TC
GintGst5.for	TCT CTA ATT AAA CTA CTT GCG
GintGst5.rev	ACC ATC CAA CCG TTA TTA TTG
GintGst6.for	TGG TCG CGG CCG AGG TAC TTC CC
GintGst6.rev	TCG TGA TTG TTA TCA CAT GTT AAG
GintTef1.for	TAC TTG ATT TAC AAG TGC GGT GG
GintTef1.rev	TTT GAC CGT CCT TGG AGA TAC C

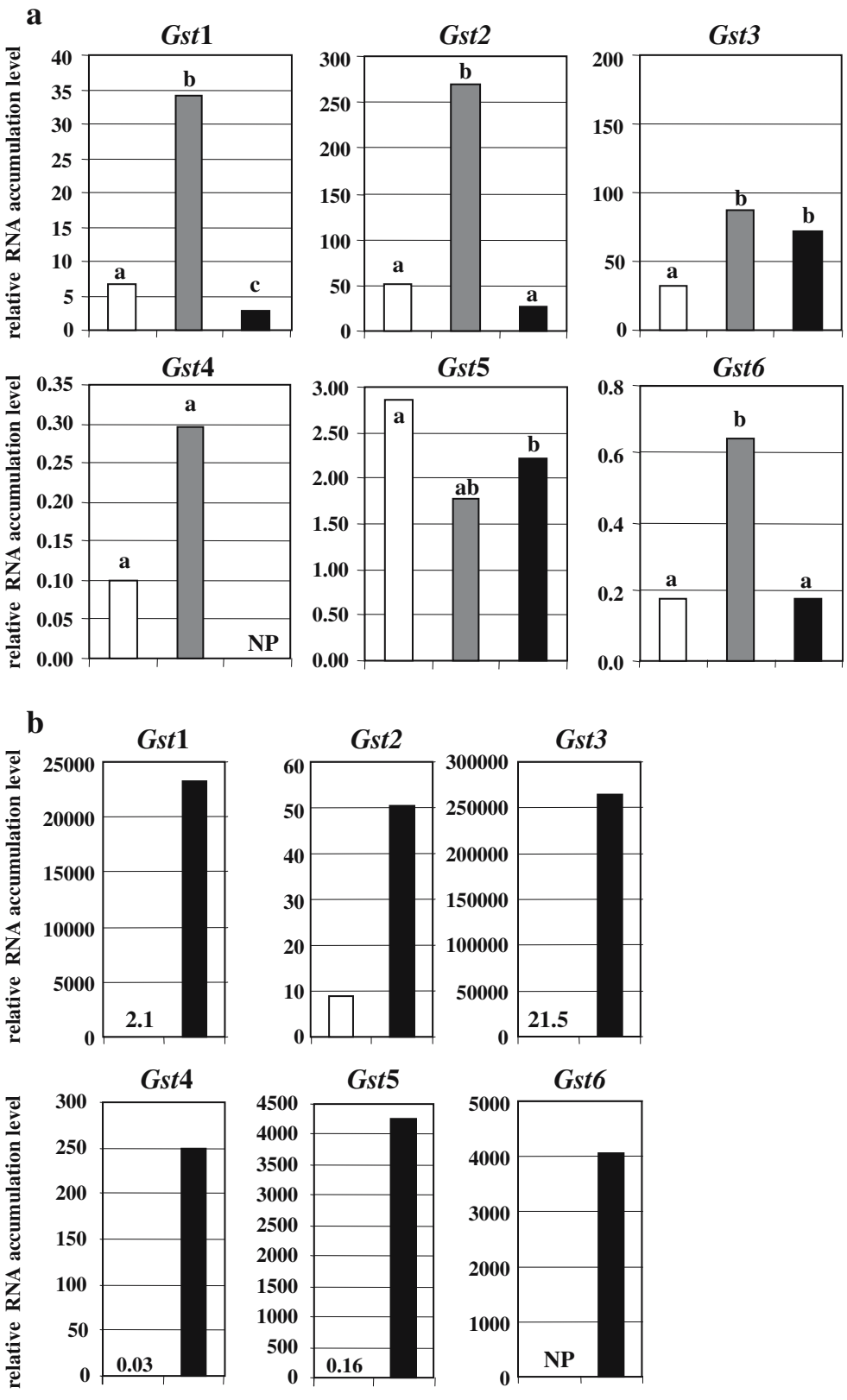
(Requena et al. 2002; Tamasloukht et al. 2003; Breuninger and Requena 2004). Reverse Northern blot analysis identified 51 ESTs that probably belong to genes induced by HMs. Two of these genes putatively encoding a thioredoxin and a GST have been identified in a similar study as being induced by Zn (Ouziad et al. 2005).

Among the ESTs of *G. intraradices* HM-induced genes, 24 showed significant similarities to sequences in the databases indicating specific cellular functions in HM-responses for the corresponding proteins. Histone acetylases are known to be involved in higher-order gene regulation (Ikura and Ogryzko 2003). They are associated with so-called nuclear bodies, which accumulate upon HM treatment (Zimber et al. 2004). This would require higher production of these enzymes and, in consequence, increased accumulation of the corresponding transcript. A direct detoxification effect has been reported for an aldo/keto reductase: ectopic expression of this enzyme, which acts on cytotoxic lipid peroxidation products, provided tolerance to HMs in transgenic tobacco plants (Oberschall et al. 2000). HM-induced expression of chaperons has been detected in different organisms. A DnaJ-like protein-encoding gene, for example, is strongly induced in bean after exposure to mercuric chloride (Chai et al. 2000). The mechanism involved could be direct binding and trafficking, as has been experimentally shown in the case of copper (Field et al. 2002), or by refolding of proteins which are denatured due to the stress conditions (Suzuki et al. 2001; Shen and Hendershot 2005). It is well known that HMs also have an effect on carbohydrate concentrations (e.g., Hemalatha et al. 1997). In this context, it was interesting to detect a *G. intraradices* gene encoding a phosphoenolpyruvate carboxykinase because this is used in vertebrates

Fig. 3 RNA accumulation of *GintGst*1–6. Real-time PCR experiments were carried out with RNA from **a** germinated spores (*white columns*), extraradical hyphae (gray columns), and mycorrhiza from *G. intraradices–M. truncatula* root organ cultures (*black columns*), or from **b** 2-week-old extraradical hyphae from a carrot root organ culture which were either treated (*black columns*) or not (*white column*) with a HM solution. Values obtained with gene-specific primer pairs for *GintGst*1–6 were calibrated by those obtained with a primer pair for the *GintTef*1 gene. Different lower case letters at the columns of **a** indicate significant differences ($p<0.05$) between means from triplicate experiments, determined by the Mann–Whitney U-test. Standard deviations in **b** were less than 20%. *NP* no quantifiable product obtained

as a sensitive biomarker for carcinogenic HMs and for the identification of cis and trans elements of HM regulation (Hamilton et al. 1998). Thioredoxin gene expression is regulated by many environmental stimuli and is also induced by HMs (Park et al. 1999; Lemaire et al. 2002). The detoxification capacity of this protein is probably based on its highly conserved dithiol motif, which helps to reverse the oxidation of proteins (Watson et al. 2004). On the other hand, thioredoxin is involved in transcriptional regulation of stress-responsive genes and in pyrimidine metabolism for DNA repair (Watson et al. 2004). Interestingly, the list of HM-induced genes from *G. intraradices* also contains a guanosine diphosphatase. This enzyme has not been described in the context of stress response up to now, but being involved in purine biosynthesis, it might be necessary in helping the cell to survive in a way similar to thioredoxin. Another protein, which has not been identified up to now as being stress-responsive, is the glyoxal oxidase. It belongs to the ligninolytic enzymes and its activity gives rise to H_2O_2 (Watanabe et al. 2001). The substrate glyoxal, however, is produced from linoleic acid by lipid peroxidation (Watanabe et al. 2001), which in turn is one cellular response to HM stress (Schutzendubel and Polle 2002). It might, therefore, be a key factor in the conversion of the primary inductor "heavy metal" to the secondary signal "reactive oxygens" (see below).

GSTs catalyze the S-conjugation of the thiol group of reduced glutathione and of electrophylic moieties of diverse hydrophobic toxicants. This results in more hydrophilic and less toxic products, which can be more easily processed further by the cellular machinery. GSTs also possess a second function of being involved in the regulation of signal transduction chains following H_2O_2 treatment (Yin et al. 2000). The analysis of *Gst* expression patterns showed that the genes respond to different stress factors in plants (Marrs 1996) and also in fungi (Veal et al. 2002). Their putative role has been substantiated in transgenic plants with overexpressed GST genes, as these plants were more tolerant to diverse stresses (Roxas et al. 2000; Ezaki et al. 2000; Takesawa et al. 2002; Yu et al. 2003). Reactive oxygen species have been proposed as a common secondary stress response to the different environmental signals


a
Gst1
Gst2
Gst3
Gst4
Gst5
Gst6
relative RNA accumulation level
NP
b
Gst1
Gst2
Gst3
Gst4
Gst5
Gst6
relative RNA accumulation level
2.1
21.5
0.03
0.16
NP

(Marrs 1996), and it was shown that HMs can also induce oxidative stress (Schutzendubel and Polle 2002). However, although H_2O_2 accumulates during AM development (Salzer et al. 1999) and could be the signal for plant GST gene induction in the symbiotic interaction (Strittmatter et al. 1996; Franken et al. 2000; Bestel-Corre et al. 2002; Wulf et al. 2003; Brechenmacher et al. 2004), it does not seem to induce the fungal GST genes *GintGst*1–6 as the corresponding transcripts do not accumulate to a higher extent in mycorrhizas compared to germinating spores or extraradical hyphae. In contrast, most of the GST genes show increased RNA accumulation in the extraradical hyphae of the 2-month-old *M. truncatula* root organ cultures compared to the other developmental stages and also compared to the 2-week-old hyphae from the carrot cultures. This can probably be explained by processes of senescence in the older mycelium because senescence has been shown to be correlated with the expression of GST-encoding genes (Kunieda et al. 2005). Whether or not oxidative stress is responsible for the induction of the *G. intraradices* genes in extraradical hyphae upon HM exposure cannot be decided from the present experiments and has to be analyzed in the future using different reactive-oxygen-generating compounds.

Because GSTs are involved in the reactions of cells to many different signals, it is not surprising that they are encoded by large gene families including, e.g., 47 members in *Arabidopsis thaliana* (Wagner et al. 2002) or 59 members in rice (Soranzo et al. 2004). They are grouped into four classes according to their sequence similarity: phi, theta, zeta, and tau. Other classes have been identified in animals and bacteria (Mannervik et al. 1985; Sheehan et al. 2001), but little is known about fungal members of this group of enzymes. Very recently, however, an overview and classification of GST and GST-like genes from yeasts and fungi in comparison to other organisms has been published based on homology screenings of different databases (McGoldrick et al. 2005). According to this classification, GINTGST6 seems to belong to cluster 2 while GINTGST1–5 groups with the phi class. Among the different clusters in plants, the phi class seems to be the most highly inducible by different stress treatments (Wagner et al. 2002). The only fungal sequence reported in this class (XP_391216) was deduced from a genomic clone of *Gibberella zeae* and expression data are not available. In contrast, among the cluster 2 enzymes, one from *Aspergillus nidulans* is enhanced by xenobiotics and oxidative stress, and confers tolerance to HMs (Fraser et al. 2002).

In summary, GST genes of many organisms are inducible by HMs, probably via the production of reactive oxygen species, and they are functional in resistance to these xenobiotics. Therefore, it might well be that the GST genes identified in *G. intraradices* are important for the survival of the fungus in HM-contaminated soils and hence play a crucial role in the important aspect of the application of AM fungi to confer HM tolerance to plants. Future research will compare expression patterns and sequences of GST genes derived from *G. intraradices* isolates with different capabilities of being bioprotectors against abiotic stress.

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